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Laura C. Hudson
University of Tennessee - Knoxville

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To the Graduate Council:

I am submitting herewith a thesis written by Laura C. Hudson entitled "The Use of Green Fluorescent Protein for Transgene Monitoring and Detection of Pollen Distribution and Gene Flow Patterns under Field Conditions.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

C. Neal Stewart, Jr., Major Professor

We have read this thesis and recommend its acceptance:

Vincent R. Pantalone, Bob Trigiano

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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C. Neal Stewart, Jr.
C. Neal Stewart, Jr., Major Professor

We have read this thesis
and recommend its acceptance:

Vincent R. Pantalone

Bob Trigiano

Accepted for the Council:

Anne Mayhew
Vice Provost and
Dean of Graduate Studies

(Original signatures are on file with official student records)

The Use of Green Fluorescent Protein for Transgene Monitoring and Detection of Pollen Distribution and Gene Flow Patterns Under Field Conditions

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Laura C. Hudson

August 2003

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Abstract

There have been many recent and new possibilities for crop improvement since the development of techniques, which allow for the transfer of novel genes into host plant genomes. Fitness enhancing genes that confer disease, drought, and herbicide resistance to important food crops such as corn (*Zea mays* L.), soybean (*Glycine max* L.), canola (*Brassica napus* L.), and rice (*Oryza sativa* L.) will be invaluable tools to feed the exponentially growing human population world wide.

This widespread use of transgenic crops has increased the risk of transgene escape into the environment. This risk becomes more significant when engineered crop species have wild relatives growing in close proximity. The potential of transgenes resulting in fitness-enhancement of weedy relatives warrants the need for an *in vivo* gene monitoring system suitable for use in the field so that the potential risks can be quantified and evaluated. Green fluorescent protein (GFP) has been demonstrated as an effective tool to monitor the expression and possible introgression of transgenes into crop-related wild species (Stewart, 1996; Harper *et al*., 1999; Halfhill *et a*l., 2001). Therefore, GFP may be an ideal candidate for the detection of gene flow and pollen distribution patterns under field conditions.

In this study, the pollen-specific LAT59 promoter was used to express a green fluorescent protein (GFP) gene in tobacco (*Nicotiana tabacum* L.*)*. The result yielded transgenic tobacco plants, which express GFP protein only within pollen grains. GFP-tagged pollen was developed as a tool for tracking the movement of transgenic tobacco pollen under field conditions. A two-year field study was set up and monitored during the summer f 2001 and 2002. The goal of this research was to characterize spatial

distribution patterns of transgenic pollen to gain knowledge about pollination mechanisms under field conditions in tobacco. However, a possible pitfall is that tobacco is normally a self-fertilizing crop so detection of pollen movement in this system may be difficult.

Another aspect of this research was to estimate the frequency of out-crossing under field conditions. Transgenic tobacco engineered with the CaMV35s promoter to express GFP throughout the entire plant (WPGFP) was used. Pollen flow from a pollen-donor population of WPGFP tobacco plants to non-transgenic tobacco plants grown at specified distances and directions from the transgenics were assessed. Hybrid frequency was determined by screening the progeny from each wild type recipient plant for the GFP phenotype. Out-crossing in 2001 was 0.009% and 0.07% in 2002. Average out-crossing within the center plot in 2002 was 0.7%. The goal of this research was to determine the dynamics of actual out-crossing events in a field of tobacco. If this system is to be an effective monitoring tool, then GFP transgenic pollen viability and longevity must be tested. Anytime a foreign protein is introduced into a reproductive cell, there is a possibility of adverse effects on cellular regulation or disruption of cell development, which could be detrimental to a tobacco plant's capacity to reproduce. Expression of GFP has been demonstrated in many different plant parts and species, and toxicity or adverse developmental events have not been observed (Pang *et al*., 1996; Leffel *et al*., 1997; Harper *et al*., 1999; Quaedvlieg *et al*., 1998; Tian *et al.,* 1999; Molinier *et al*., 2000). Although GFP expression has not been found previously to have an effect on plant cells, a comparison of pollen tube germination frequencies to those of wild type tobacco pollen

were conducted to determine the consequences of GFP expression. Data from these experiments did not suggest that expression of GFP had an effect on pollen fitness.

Since GFP can be expressed in tobacco pollen under the control of the LAT59 pollen specific promoter, a system to monitor and detect pollen distribution and gene flow patterns can be developed on a large scale. This could reveal answers to many questions involving ramifications of the introgression of transgenic crop species into the environment.

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Chapter One

Literature Review

Potential Impacts of Biotechnology:

Perceived threats of uncontrollable transgene spread into the environment has lead to controversy about the introduction of transgenic plant species into agricultural practices. However, genetically modified plants are becoming rapidly incorporated into our agricultural system. Crops such as corn (*Zea mays*) canola (*Brassica napus*), tomato (*Lycopersicon esculentum* L.), potato (*Solanum tuberosum* L.), soybean (*Glycine max*), cotton (*Gossypium hirsutum* L.), and rice (*Oryza sativa*) have been genetically modified with various enhancement genes. Introgression of these crops into the environment could lead to the movement of transgenes to wild relatives, which raises the concern of their potential impact on the environment and non-target species (Losey *et al.,* 1999; Sears *et al*., 2001). Concerns are also raised when a new genetically modified product or agricultural commodity enters the food supply. Several transgenic foods have been evaluated for safety for human consumption, and a number of products are available on the market, however, there is concern as to whether the food is safe for consumers.

Genetically modified crops cover millions of acres across the United States, where large quantities of GM foods are consumed. Until recently, there has been little public debate until the monarch butterfly (*Danaus plexippus*) became the subject of significant scientific and media interest regarding non-target effects from biotech crops. This issue

focused on the pollen of transgenic corn that could blow onto milkweed (*Asclepias tuberosa* L.) leaves the exclusive diet of monarch caterpillars.

Bacillus thuringiensis (Bt) is a naturally occurring soil borne bacterium, which produces a crystal like protein (Cry proteins) that selectively kills a specific group of insects (Lepidopteron, the caterpillars) and is not harmful to agriculturally beneficial insects (MacIntosh *et al*., 1990; Pilcher *et al*., 1997) animals or humans (McClintock *et al.*, 1990) and does not persist in the environment (Sims and Holden, 1996). Liquid and granular formulations of the Bt proteins have been used successfully for 30 years on a variety of crops. Bt corn refers to corn that has been enhanced through biotechnology to produce its own Bt insecticidal proteins.

Regulation for Bt corn and other transgenic crop species falls under the jurisdiction of the Environmental Protection Agency (EPA) in the United States, that is responsible for determining environmental impacts. Before the approval of Bt corn the EPA concluded that the product does not cause any "unreasonable adverse effects" to non-target organisms (EPA, 1995; EPA, 1996; EPA, 1998) based on evaluations of toxicity and exposure.

In May 1999, a communication in the journal Nature (Losey *et al*., 1999) raised concerns regarding non-target effects of transgenic crops containing genes from *B. thuringiensis*. A laboratory feeding experiment performed by researchers at Cornell University found monarch butterfly larvae that were given no choice but to fed on milkweed leaves dusted with high levels of pollen from Bt corn had slower growth rates and a higher morality rate than those larvae consuming leaves with no pollen or non-Bt pollen. This group of scientist suggested that there was a potential risk to monarch

butterfly populations, if demonstrated in the natural environment, since migratory patterns include the central United States, where the majority of corn is grown.

In order to address public concerns about the monarch, researchers from nine universities, the U.S. Department of Agriculture, and Agriculture Extension conducted numerous field test in 1999 and reported their findings at the Monarch Butterfly Research Symposia. This collaborative work found that monarch larvae have very little exposure to corn pollen under field conditions (Sears *et al*., 2000a; Wraight *et al*., 2000) since the majority of corn pollen settles in the immediate vicinity of the field. Pollen movement is limited; however wind can disperse pollen away from the field, depositing it over a broad area creating low pollen concentrations on surfaces. Further field studies demonstrated that milkweed leaves captured only 30 percent of corn pollen available and wind and rain can reduce this amount another 90 percent (Dively *et al*., 2000). Bt corn pollen with exposure to sunlight for 8 days had little activity on monarch larvae due to degradation of the active protein by the ultraviolet light (Head and Brown, 1999).

Monarch butterflies may have exposure to small quantities of Bt corn pollen on milkweed, but at amounts below the threshold to harm the larvae. Monarch larvae can safely consume milkweed leaves with up to 1100 Bt pollen grains per square centimeter (Hellmich *et al*., 2000a), which is high for field levels. Surveys were conducted in 81 Maryland fields showing the mean pollen level inside of the cornfields or around the edge was 56 pollen grains and only 10 leaves out of 127 leaves examined had higher levels (Dively *et al*., 2000). Monarch larvae would avoid eating milkweed with Bt or non-Bt corn pollen on the surface if milkweeds free from pollen were available (Hellmich *et al*., 2000a).

More recent field studies conducted in Canada and Iowa and sponsored by the USDA and the Agricultural Biotechnology Stewardship Technical Committee concluded that monarch survival, weight gain, and milkweed consumption were similar to monarch larvae feeding for five consecutive days on milkweed plants in Bt and non-Bt cornfield during pollen shed (Sears *et al*., 2000b; Hellmich *et al*., 2000b). These experiments confirmed that milkweed leaves in close proximity to Bt cornfields contained pollen level too low to impact the normal development of the monarch butterfly larva.

Now that the question of an immediate significant risk has been answered, studies are now under way, according to the USDA, to determine if there are subtle effects on the larvae. Caterpillar exposure to Bt corn pollen for extensive time periods and whether older caterpillars consume anthers that fall onto milkweed leaves unintentionally since Bt protein levels were found to be higher in these plant organs would be investigated. Many factors contribute to fewer than 10 percent of monarch caterpillars making it to adulthood and should be weighed in comparison to the mortality rate with which they are associated. Furthermore, future conclusions addressing environmental impacts on non-target organisms by transgenic crops needs to be approached with appropriate scientific methods and assessment procedures.

Non-target effects of transgenic plants are not the only topics portrayed in the media headlines. Products of biotechnology should be no different than conventional foods, however, they are more heavily scrutinized than their traditional counterparts due to public reservations and confusion over recombinant DNA technology. Transgenic foods will be nearly identical to their predecessors except for the changes introduced by

biotechnology, nonetheless genetically engineered foods are rigorously evaluated for toxicity and allergenicity before approval for consumption.

Starlink® corn, commercialized by Aventis CropScience, USA, has been modified through well recognized genetic techniques to produce the protein Cry9C for its known insecticidal properties from the bacteria *B. thuringiensis*. Cry9C is a variant of a number of Bt toxins including the commercially used Cry1A mentioned previously. Starlink® corn was registered in 1998 for industrial uses and animal feed with the EPA under the Federal Insecticide, Fungicide, and Rodenticide Act. The EPA concluded, after granting the registration of Starlink®, that the Cry9C protein met the safety standard for use in field corn for animal feed based on the toxicology data and limited exposure expected with animal feed use and that there was reasonable certainty that no harm would result in exposure to the human population (EPA, 2000). The EPA did not extend the exemption to human food because there was concern, according to the CDC, that the Cry9C protein shares several molecular properties with proteins that are known food allergens although the incidence of food allergies in the human population is only 1% for adults and 5% for infants (FDA, 1997).

On September 18, 2000 a press conference was held announcing that taco shells purchased from a local grocery store contained trace amounts of GM corn DNA associated with Starlink®. This event was convened by Genetically Modified Food Alert, a consortium of seven consumer organizations based out of Washington, D.C. who notified Kraft foods of their findings. A few days later Kraft Foods announced its voluntary recall of Taco Bell Home Originals taco shells and taco dinners sold nation wide, which resulted in the return of 2.5 million boxes of taco shell products. Following

Kraft's action a number of other food manufactures issued recalls for products made from corn resulting in the eventual recall of nearly 300 food products.

An investigation done by the CDC established that 28 of the people who filed adverse event reports after eating a product containing the Cry9C protein had experienced an allergic reaction. However, their study could not confirm a link between Cry9C and the production of detectable amounts of the Cry9C-specific antibody in blood serum from the patients. The CDC stated that although their results did not provide evidence that the allergic reactions experienced by these people were associated with the Cry9C protein, the possibility could not be completely ruled out leaving the EPA with the responsibility to decide how to regulate plants containing the Cry9C protein (CDC, 2001). Following this string of events, Aventis stopped the sales of Starlink® seed and agreed to purchase Starlink® corn in order to isolate it form the US food supply. A few months later Aventis announced that is was canceling the registration of Starlink® corn resulting in the corn being banned for any agricultural purpose.

The Consumers Union of Japan reported that Starlink® protein had been detected in cornmeal and exports to Japan, the largest foreign market for US corn, dropped by about two-thirds while South Korea, the second largest consumer of US corn, banned it all together. Many proposals for reform surfaced in light of the Starlink® recalls, including legislation making it mandatory for the FDA to review GM foods (S3184: The Genetically Engineered Foods Act of 2000) and a promise to reintroduce a bill to establish one agency with primary responsibility for food safety. The issue of food labeling was also raised after this controversy however, the FDA reaffirmed its decision to not require special labeling of all bioengineered food, but proposed guidelines on

voluntary labeling. Since genetically modified crops are conceptually different from the technologies employed before with traditional plant breeding, the question becomes what new complications and risks are posed by biotechnology and how can they be effectively managed to allow its safe use now and in the future.

The Need for a Monitoring System:

To address some of these concerns, a model system needs to be created in order to assess pollen migration patterns in the field. This system needs to be simple, rapid, and suitable for use on a large scale while remaining cost effective. It has been proposed to use whole-plant expressing GFP to monitor gene flow under agricultural conditions (Stewart, 1996; Leffel *et al*., 1997; Harper *et al*., 1999). Currently, there is no system for direct monitoring of pollen movement. One possibility may be GFP-tagged pollen used to monitor pollen flow directly under field conditions. There are several basic and applied applications of GFP-tagged pollen. Spatial distribution patterns of pollen and amounts of pollen flow from a pollen-donor population of transgenic plants to specified distances and directions could be estimated by tracking the movement of pollen through the environment.

While GFP-tagged pollen will benefit risk assessment research in the near-term, such a system might be an enabling tool in the commercial monitoring of transgenic crop pollen flow. One example would be to track pollen flow from transgenic to organically grown fields containing the same crop. Likewise, pollen containment could be assessed using traps and pollen movement via wind and insects could be documented. The detection of pollen distribution and gene flow patterns using GFP will lead to effective

risk assessment, which will improve upon implementation and management of biotechnology. This will ultimately enhance the safe introduction and use of genetically modified crop species.

Green Fluorescent Protein as a Marker:

Green fluorescent protein (GFP) was isolated and cloned in 1992 from a bioluminescent jellyfish, *Aequorea victoria.* GFP consists of 238 amino acids with wild type excitation peaks of 395nm and 475nm and an emission peak of 508nm (Chalfie *et al.*, 1994). GFP is a thermostable protein with a structure of cylindrical walls formed from 11 strands of β-sheets capped on top and bottom with short segments of α-helices forming a 27 kDa monomer. This scaffolding houses a chromophore near the geometrical center (Cody *et al*., 1993), which is composed of three residues: serine65, tyrosine66, and glysine67 (Yang *et al*., 1996). Several variants have been developed whose modifications have created a higher level of fluorescence, improved expression in eukaryotic cells, and altered spectral properties (Haseloff *et al*., 1997; Siemmering *et al*., 1996). The native GFP gene contained a cryptic intron, which caused splicing in plant cells between the nucleotides 380-463 that created an 84-nucleotide intron leading to reduced fluorescence. This intron has been mutated by modifiying the codon usage of the gene to restore proper expression (Haseloff *et al.,* 1997).

GFP is normally found within the nucleoplasm and cytoplasm of a transformed cell, however targeting GFP to the endoplasmic reticulum (ER) of a cell ensures secretion and retention of GFP within the lumen of the ER. This targeting form of GFP (mGFP5-ER) has a signal peptide, derived from *Arabidopsis* vacuolar basic chitinase, which allows for

higher concentrations of GFP in transformed cells and stronger visible expression (Haseloff *et al*., 1997). In addition, this ER targeted form of GFP (mGFP5-ER) was mutated with site-directed mutagenesis forming two mutants with improved folding patterns leading to greater efficiency when exposed to high temperatures (Siemmering *et al*., 1996). These mutations have improved the thermal stability of GFP and have made it suitable for use as a visual marker.

GFP is a valuable tool used to assess frequency of stable transformation during tissue culture and in monitoring the gene flow of transgenic plants in the environment (Stewart, 1996). GFP does not require a co-factor for fluorescence, which makes it an effective, noninvasive, *in vivo* marker for gene expression (Leffel *et al.*, 1997), which can be detected in a nondestructive manner (Halfhill *et al*., 2001). The variant mGFP5-ER has been shown to withstand field conditions and is strongly visible in plant cells (Harper *et al.,* 1999).

Pollen Specific Promoter:

The tomato LAT59 promoter (Twell *et al.,* 1989) is a pollen-active promoter, which controls the expression of a gene that shows significant similarity in amino acid sequence to the pectate lyases of the plant pathogen *Erwinia*. The LAT59 promoter is preferentially expressed in the anthers and pollen of tomatoes (Twell *et al.*, 1989). Transcription from the LAT59 promoter has been found to occur post-meiotically and regulation appears to be co-ordinate during the development of anthers (Twell *et al*., 1990). The LAT59 proteins have also been found on the surface because they are secreted and glycosylated (Dirks *et al*., 1996). Plant members belonging to this family

might play a role in cell wall deposition as the pollen tube grows through the pistil (Kulikauskas and McCormick, 1997) and have distinct N-terminal extension (Kim *et al*., 1994).

The Lat59 promoter was chosen for this study over the CaMV35s constitutive promoter due to its sufficient expression of GFP in tobacco pollen (Twell *et al.*, 1989). Transgenic plants expressing GFP under the CAM35s promoter did not show signs of GFP expression within pollen cells of tobacco when viewed under a fluorescent microscope.

Certain constitutive promoters such as the ubiquitin and actin promoters have been shown to express GFP throughout the entire plant as well as the pollen cells and could be good candidates for further experiments (Christensen and Quail, 1996; Richards *et al.*, 2001). However, at this time, a set of data is needed to distinguish between the WPGFP tobacco and the pollen specific tobacco according to their location within the field plot.

Target Species:

Gene flow in tobacco is thought to be low considering that it is characteristically a self-fertilizing crop. The anthers discharge the pollen before or soon after the flower opens allowing the pollen to fall directly onto the stigma. In most states isolation distances of approximately 133m is considered adequate for the production of certified seeds. However, out crossing events in tobacco have been documented to occur up to 727m (McMurtrey *et al.*, 1959) therefore, other precautions must be taken to ensure the production of pure seed. Even though tobacco farmers go to great lengths to prevent the

flowering of tobacco that they will use for sale, flowering can occur quickly, increasing the risk of gene flow and making it a candidate for this study.

Canola *(Brassica napus)* is a better candidate considering that it is a known out-crosser via wind and insects. When canola flower petals unfold pollen is shed and flowers remain receptive for up to three days after opening. Canola pollen is heavy, sticky, and falls directly onto the plant surface or ground. Canola is predominantly an inbreeding crop with a high percentage rate of self-fertilization. However, a small percentage of canola pollen can become air borne and travel via wind, allowing out crossing to occur. This leads to isolation distances for purity of pedigree seeds that is much larger (approximately 800m) than tobacco. Transgenic canola may also be problematic since transgenes can flow to weedy wild relatives.

Gene Transfer into Target Species:

In order to develop a gene flow monitoring system using a visual marker, transgenic tobacco plants must be created that express the GFP protein. This will be possible by using a peculiar type of bacteria. In addition to their main chromosome, many bacteria possess large numbers of small circular DNA molecules called plasmids. Plasmids are autonomously replicating bacterial DNA molecules with the capacity to deliver a specific genetic sequence into a plant cell. The sequence is incorporated into the genome of the cell, resulting in a stable transformation. A transgenic plant can be obtained through tissue culture practices, which trigger the induction of cell division and differentiation into organs such as shoot and root meristems.

Several methods exist and have been used for gene delivery into plant cells. One of the most commonly used and successful methods for the transformation of dicotyledonous species is *Agrobacterium* mediated transformation. This method utilizes a pathogenic bacterium species called *A. tumefaciens,* which is found in soil across the world and is responsible for the tumorous growths known as crown gall disease. Gall formation results from the accelerated cell divisions initiated in the infected plant tissue caused by genes transferred from the *Agrobacterium* to the cell on a piece of plasmid DNA known as transfer DNA (TDNA). The transfer DNA can be modified by removing the tumor-inducing and opine genes which lie between the boarder regions of the plasmid and replacing them with a recombinant transgene (Fraley *et al*., 1986). Plant cell are infected with the engineered *Agrobacterium* strain, which incorporates the desired genes into the host plant genome resulting in transgenic tissue. This method has been used to genetically modify a variety of dicotyledonous plants including several *Solanaceae* species and forage legumes successfully (Figueria-Filho *et al*., 1994; Voisy *et al*., 1994; Kumar *et al*., 1995; Ninkovic *et al.,* 1995). However, success with *Agrobacterium* transformation on monocotyledonous species has been minimal. This could be attributed to the fact that monocotyledonous plants are not natural hosts for the bacteria, and therefore experience less efficient gene transfer and integration (Christou, 1995). This method is more difficult in cereal or grass transformation systems although transformation with *Agrobacterium* has been demonstrated on maize, rice, and barley (Hiei *et al*., 1994; Ishida *et al*., 1996; Tingay *et al*., 1997).

Chapter Two

GFP-tagged Pollen to Monitor Pollen Flow of Transgenic Plants

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This chapter is a paper published in the journal *Molecular Ecology Notes* in 2001 by Laura Hudson , Dean Chamberlain and Charles Neal Stewart, Jr.:

Hudson LC, D Chamberlain, CN Stewart, Jr. (2001) GFP-tagged Pollen to Monitor Pollen Flow of Transgenic Plants. *Molecular Ecology Notes*, **1**, 321-324.

Abstract:

In this study, the pollen-active LAT59 promoter from tomato was used to express a green fluorescent protein (GFP) encoding gene in *Nicotiana tabacum* (tobacco) pollen. This promoter is preferentially expressed in anthers and pollen. Pollen in transgenic plants segregated in a 1:1 Mendelian ratio, and the plants were PCR-positive. GFP-tagged pollen was developed as a tool for tracking the movement of transgenic plant pollen in the environment. Specifically, it should be a useful tool for characterizing the spatial distribution patterns of transgenic pollen, to determine pollination mechanisms, to monitor the effects on non-target organisms, and to monitor gene flow in field conditions.

Introduction:

As the result of the widespread use of transgenic crops, the potential escape of transgenes into the natural environment has become a growing concern. Since some

engineered crop species have wild relatives growing in close proximity, the risks of transgene escape causing fitness-enhancement of weedy relatives warrants the need for an *in vivo* gene monitoring system suitable for use in the field. Another concern is non-target risks, such as side effects of Bt-transgenic pollen on beneficial or ecologically important insects. An important tool for monitoring possible introgression of genes such as herbicide, disease, insect, and drought resistance into weedy relatives is green fluorescent protein (GFP) (Stewart, 1996). The gene encoding green fluorescent protein (GFP) was isolated and cloned from the jellyfish *Aequorea victoria*, and several spectral variants have been developed. GFP is a 27 kDa monomer that fluoresces green under long wave ultraviolet or blue light. (Chalfie *et al.*, 1994). GFP does not require a co-factor for fluorescence, which makes it an effective *in vivo* marker of gene expression (Leffel *et al.*, 1997). GFP is a valuable tool used to assess frequency of stable transformation during tissue culture and in monitoring the gene flow of transgenic plants in the environment (Stewart, 1996). This paper demonstrates that pollen tagging with GFP is a viable and useful approach to visualize transgenic pollen for tracking.

In this study, the tomato LAT59 promoter (Twell *et al.,* 1989) was used to express the variant *mgfp5-er* in *Nicotiana tabacum* (tobacco) pollen and visualized on honeybees. This pollen-active promoter controls the expression of a gene that is a member of a family of pectate lyase-like proteins, which is preferentially expressed in the anthers and pollen of tomatoes (Twell *et al*., 1989). GFP-expressing pollen under the control of pollen promoters were not found to impair reproductive function of tobacco (Ottenschlager *et al*., 1999). The expression of GFP in the pollen of plants will enable scientists to track the movement of pollen, to differentiate between pollen from individual

plants of the same species, to determine pollination mechanisms, and to study spatial patterns of pollen with respect to a plants location in the field. We describe proof-of-concept experiments showing the potential of such a system.

Materials and Methods:

The CaMV35s promoter cassette from the *Agrobacterium tumefaciens* expression vector pBINmGFP5-ER (Haseloff *et al*., 1997), was excised by a HindIII and BamHI restriction digest. The LAT59 promoter was ligated into the vector to replace CaMV35s promoter. The plasmid was renamed pBINDC1 (Figure 1). This vector contained an NPTII cassette (kanamycin resistance) that was under the control of the nopaline synthase promoter and terminator.

Nicotiana tabacum cv 'Xanthi' was transformed with pBINDC1 using the *Agrobacterium*-mediated leaf-disc transformation method (Horsch *et al*., 1985). Transgenic plants were selected on MS media (Murashig and Skoog, 1962) containing kanamycin (200mg/l) and timentin (400 mg/l). Shoots arising from leaf discs were rooted on agar solidified MSO medium (McCormick *et al.*, 1986). After the plantlets formed roots, they were transferred to soil and grown to maturity under growth chamber conditions. Upon maturation, the plants were examined with a hand held long wave ultraviolet light (UVP model B-100AP 100 W: 365nm) to test for the constitutive expression of GFP. Pollen was collected and observed under an epifluorescent (FITC filtered) microscope with blue light to determine if pollen was expressing GFP.

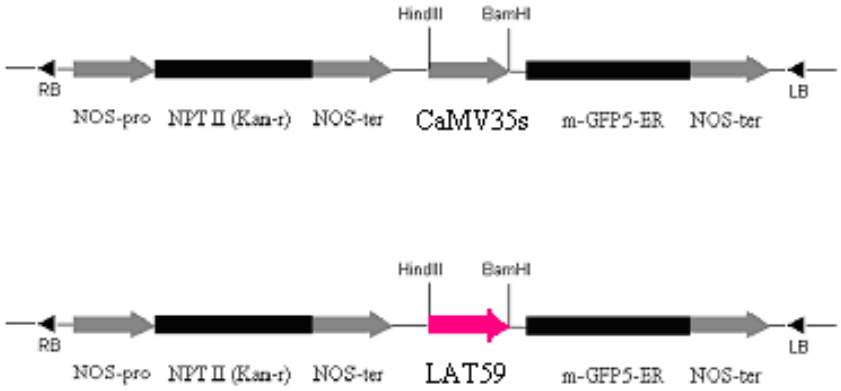

Figure 1. Construction of pBINDC1 plasmid. The LAT59 pollen-active promoter was subcloned into the *Agrobacterium tumefaciens* expression vector pBINmGFP5-ER in the place of the CaMV35s promoter to create pBINDC1 as shown.

To verify the transgenic status of the plants, PCR analysis was performed using GFP-specific primers. DNA was isolated from transgenic plants (Stewart, 1997) and the GFP fragment was amplified using PCR under conditions of 95ºC for 5 minutes, 54ºC for 1 minute, and 72ºC for 2 minutes with the forward primer 5'-CCTTAAGGATCCAAGGAGATATAACAATGA-3' and reverse primer 5'-CCGGTTGAGCTCTTAAAGCTCATCATGTTT-3'.

Transgenic flowering plants were placed in a cage with 24 bees for 7 days. Bees were captured in petri dishes and placed in a -20ºC freezer. Bees were placed on glass slide and examined under an epifluorescence microscope with a FITC filter set to determine the presence of GFP pollen on wing, head, and leg areas.

Results:

Out of approximately 100 leaf disks, coincubated with *Agrobacteria*, 12 transgenic plants were recovered. The 12 plants were morphologically identical to untransformed control plants except for green fluorescent pollen. DNA isolated from each transgenic plant was PCR positive for the GFP sequence (Figure 2). One line of trangenic plants were screened under ultraviolet light to determine if leaf, stem, root, seeds, or pollen areas were expressing GFP. The transformants exhibited no green fluorescence other than in pollen, which would suggest that the GFP protein is not being expressed (data not shown). Transgenic and nontransgenic pollen grains are identical under white light. In contrast, when GFP is expressed in plants under the control of 35s promoter, green fluorescence is evident under ultraviolet light in shoots and flowers but not pollen (Harper *et al*., 1999; Harper and Stewart 2000).

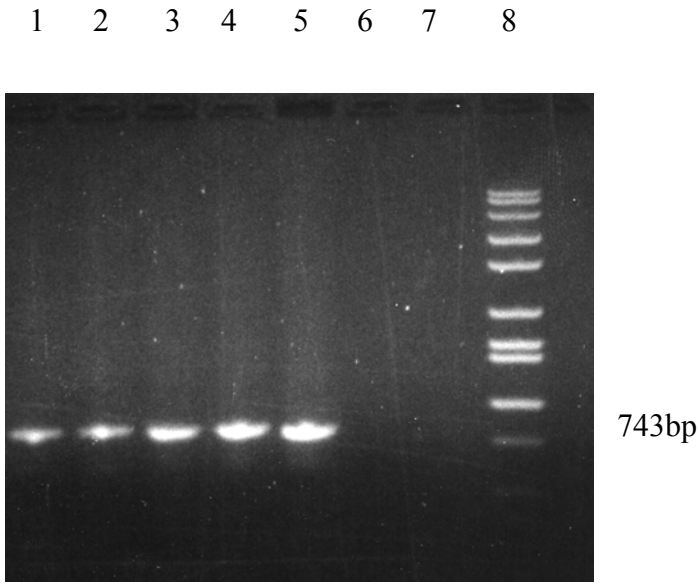

Figure 2. PCR analysis of transgenic tobacco. Lane 1 contained a positive control of purified mGFP5-ER plasmid. Lanes 2-5 contained genomic DNA from the putative transgenic tobacco plants. Lanes 6 and 7 contained the negative controls of purified water and control tobacco respectively. Lane 8 contained a high-low marker. The 743 bp band shared by lanes 2-5 illustrates that the transgene was present in the genome.

Examination of 180 pollen grains from a LAT59-GFP transformed plant showed that 96 pollen grains contained the GFP gene. Under the goodness-of-fit Chi-squared test a P value of 0.31 indicated that a 1:1 Mendelian ratio of the pollen grains expressed GFP (Figure 3A). This resulted in the insertion of the GFP gene on 1 chromosome. It is possible for the GFP gene to insert into many different chromosomes; in this case the result would not be a 1:1 ratio. Transgenic pollen on honeybees (*Apis mellifera*) leg and head areas were easily identified (Figure 3B)

Discussion:

There is great controversy about the introduction of genetically modified plants into the environment. One important issue is the movement of transgenes from crops to wild relatives. We have proposed using whole-plant expressing GFP to monitor gene flow in the field (Stewart, 1996, Leffel *et al*., 1997, Harper *et al*., 1999). However, GFP-tagged pollen may be used to monitor pollen flow directly and in mixed populations and communities; there is no current system for direct monitoring of pollen movement. This study demonstrates that the visualization of GFP pollen is possible in nature.

There are several basic and applied applications of GFP-tagged pollen since GFP can be seen in real time and can be used in large-field level situations non-destructively. Systems that are currently being used to determine pollination mechanisms are ineffective in the field because of the small size and mobility of pollinators and pollen. Highly variable markers such as microsatellites are available for some plant species but PCR is laborious and does not provide *in vivo* and real time results. Systems such as pollen traps to monitor wind pollination cannot differentiate between plants of the same

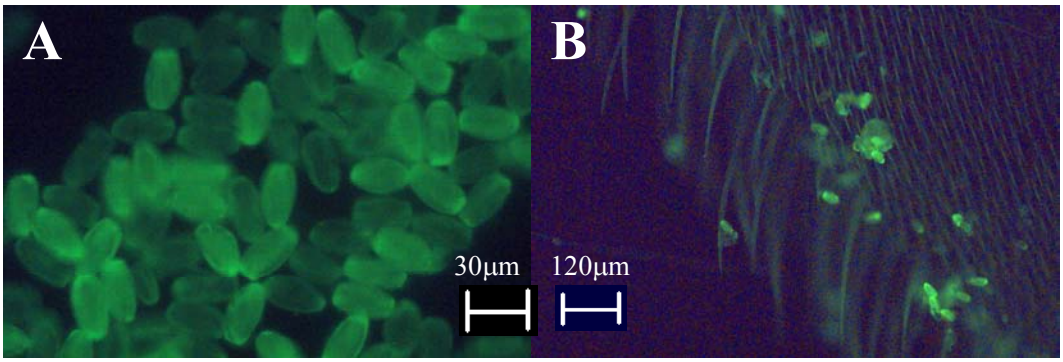

Figure 3. *Nicotiana tabacum* (tobacco) cv 'Xanthi' transgenic pollen shown under epifluorescence microscopy using blue light excitation (FITC filter set). (A) 400x magnification demonstrating 1:1 Mendelian segregation. (B) 100x magnification showing transgenic and nontransgenic segregating pollen on a bee leg.

species. Although, the use of cytoplasmic male sterile (CMS) plants can provide an indirect method to differentiate between transgenic and non-transgenic pollen from the same species, GFP-tagged pollen provides a much more efficient method. It is feasible that noninvasive monitoring of the presence of GFP-tagged pollen on insects could be done automatically using photonic systems. Therefore, we could use this system to learn more about the pollinator/pollination system.

When a pollinator, such as a bee, is introduced to the flowers of a plant expressing GFP pollen, the movement of the pollen can be tracked throughout the environment. Pollinators could be used to estimate the amount of pollen flow from a pollen-donor population of transgenic plants to non-transgenic plants grown at specified distances from the transgenics.

While GFP-tagged pollen will benefit risk assessment research in the near-term, such a system might be an enabling tool in the commercial monitoring of transgenic crop pollen flow. One example would be to track pollen flow from transgenic to organically grown fields containing the same crop. Likewise, pollen containment could be assessed using traps and pollen movement via wind and insects could be documented.

Further studies will involve an analysis of the progeny from the first line of plants obtained and a comparison of the strength of this pollen promoter to other pollen specific promoters. We will also begin pollen distribution and gene flow monitoring experiments using canola (*Brassica napus*) as a model crop in the field under a variety of conditions.

Chapter Three

Effects of Green Fluorescent Protein on Pollen Fitness

Abstract:

The male gametophyte in plants is an attractive experimental system for applications in molecular biology such as markers for gene expression, developmental processes, reproduction, and risk assessment of transgenic crops. Genetically engineering pollen with visible marker genes could open up new research possibilities for risk assessment such as use in a monitoring system for tracking the movement of transgenic pollen. In order for GFP to be effectively used as a marker in pollen, studies must be performed to see if GFP expression within pollen cells poses any fitness related advantages or disadvantages when compared to unaltered pollen.

In this study, we measured the pollen fitness of *Nicotiana tabacum* cv 'Xanthi' by comparing average pollen tube germination frequencies and pollen tube growth rates *in vitro*. Pollen from three lines of tobacco plants was compared: First, plants expressing GFP in pollen cells only; Second, plants expressing GFP under the control of a constitutive promoter (CaMV35s); and third, Wild type plants. Pollen expressing the GFP protein did not differ significantly in average pollen germination frequencies to pollen without GFP ($P = 0.65$). Average pollen tube growth rates in mm/hr over a 5 hour period did not show significant differences between transgenic and non-transgenic types (R^2 of GFP-tagged, 35s-GFP, and wild type were 0.89, 0.98, and 0.95 respectively). Overall, GFP expression in pollen grains of tobacco did not have an effect on pollen

fitness under the controlled experimental conditions of this study. This is important because the success of using green fluorescent protein as a marker in pollen would be greatly affected if its expression had fitness costs to pollen.

Introduction:

The male gametophyte of a flowering plant is a highly specialized tissue that is essential for plants reproductive success. A mature pollen grain is responsible for the recognition of a compatible stigma and delivery of sperm nuclei to the plant ovule (Cheung, 1996). Tobacco pollen is essentially a sporophyte-independent binuclear structure originating from two meiotic and one mitotic division. A tobacco pollen grain consists of only two cells, a vegetative nucleus and a generative cell, surrounded by a relatively thick exine cell wall (McCormick, 1993). The rise of the haploid microspore after the first two meiotic divisions is considered the start of plant gene expression and development, which makes the male gametophyte an attractive experimental system for various issues in plant molecular biology.

Binucleate pollen is the developmental stage of the male gametophyte of higher plants that has undergone one nuclear division after meiosis. Depending on whether or not anther dehiscence occurs after the first or the second mitosis, mature pollen is designated binucleate or trinucleate respectively. Tobacco pollen is binucleate and an ideal model to use for the study of pollen tubes. Techniques for *in vitro* and *semi vivo* germination of pollen have been well established in the tobacco system (Mulcahy and Mulcahy, 1985) and elongated tubes can be easily obtained. *Arabidopsis thaliana*, and some *Brassica* species are members of the 30% of plants species who have trinucleate pollen and do not

germinate well in *in vitro* environments (Preuss *et al.,* 1993). *In vitro* growth of most trinucleate pollen types subsides in less than 1 hr however binucleate pollen can grow in excess of 5 hours (Hoekstra, 1979).

Although *in vitro* germination provides a controlled experimental environment, it does have limitations. Germination *in vitro* does not completely mimic growth *in vivo*. Under highly optimum germination conditions, *in vitro* pollen tubes only reach approximately 30 to 40 % of *in vivo* lengths (Read *et al*., 1993). While it may not be possible to duplicate the interaction between the pollen and the pistil, in many species including tobacco, pollen germination and tube growth are robust under experimentally defined conditions, rendering *in vitro*-based studies of relevance to the *in vivo* situation (Taylor and Hepler 1997).

The expression of genes encoding green fluorescent protein (GFP) within cells has many applications and has stimulated experiments that could not have been imagined ten years ago. GFP has been used as a molecular marker in many organisms (Chiu *et al*., 1996; Muldoon *et al*., 1997) as has been optimized for use in plant systems (Reichel *et al*., 1996; Chui *et al*., 1996; Pang *et al*., 1996; Davis and Vierstra, 1998; Haseloff *et al*., 1997; Haseloff, 1999). The expression of GFP in pollen has several applications such as use as a marker for gene expression, pollen development (Benedikt *et al*., 1998), reproductive biology (Ottenschlager *et al*., 1999) and a marker for pollen distribution patterns for risk assessment under field conditions (Hudson *et al*., 2001).

The length of pollen viability after dehiscence is crucial to successful pollination (Stone *et al*., 1995) due to the fact that it determines the possibility of out-crossing among and between species. With the rapid development of biotechnology, many transgenic

crops are becoming available, resulting in biosafety concerns about possible ecological risks such as interspecific or intraspecific transgene escape. The evaluation of transgene introgression becomes an important issue for breeders, environmentalists, and policy makers. Studying the viability of transgenic pollen is important in evaluating out-crossing frequencies under field conditions.

In order to use genetically engineered pollen for risk assessment studies, there is a need to assess the effects of recombinant foreign proteins in reproductive cells. The presence of the GFP transgene in pollen could lead to problems with regulation or development. Factors such as transgene location could cause insertional effects in DNA leading to mutations. Excess protein expression of could lead to competitiveness and poor use of resources. Cytotoxicity caused by the expression of GFP, which has been documented in mammalian cells (Liu *et al*., 1999), could have adverse affects on regulation or disruption of pollen cell development and could ultimately lead to cell death. The goal of this investigation was to examine any fitness costs caused by the presence of GFP expression in pollen cells of *Nicotiana tobacum* cv 'Xanthi' when compared to tobacco pollen without GFP. In order to answer this question pollen tube germination frequencies and growth rates between tobacco pollen expressing GFP under the control of the tomato LAT59 pollen specific promoter (GFP-tagged), pollen from tobacco expressing GFP under the control of the CaMV35s constitutive promoter (35s-GFP), and wild type tobacco pollen *in vitro* were compared.

Materials and Methods:

Description of plant material

In order to test the effects of GFP on pollen fitness, all pollen produced from GFP-tagged pollen tobacco plants (pollen from plants that are expressing GFP under the control of the LAT59 pollen specific promoter (Hudson *et al*., 2001)) had to be expressing GFP. Seeds were collected from the T_0 generation were sterilized with a 70% ethanol solution for 1 minute, 20% bleach with 0.001% Tween20 for 8 minutes and washed 3 times with sterile distilled water. Seeds were grown on MS medium (Murashige and Skoog, 1962) containing kanamycin at 200mg/L for selection to produce T_1 plants. This process was repeated until seedlings from the T_3 generation were produced. These T_3 tobacco plants were homozygous for the GFP loci and 5 homozygous plants from a single transgenic event were used in this study.

Five plants that were expressing GFP under the control of the CaMV35s constitutive promoter were also analyzed for fitness. These plants express the GFP gene constitutively, however, with this promoter GFP was not expressed in the pollen of the transgenic tobacco. Five wild type tobacco cv. 'Xanthi' plants were also analyzed as a negative control.

Pollen germination frequency

Pollen from GFP-tagged tobacco plants, 35s-GFP plants, and wild type cv. 'Xanthi' was extracted during anthesis at 9 am for 5 days by tapping flowers directly above petri dishes. Pollen grains from petri dishes were placed in Falcon plate wells and 100µl of BK media (Brewbaker and Qwack, 1963) was added to 3 replicates per plant (one

replicate per well). The Falcon plate was placed in an incubator at 25ºC for 3 hours. After 3 hours 15µl of pollen grains in BK media were placed on microscope slides. Pollen tubes were counted at 100X magnification under a microscope. Using a digital camera (Olympus Q color 3) the image was projected onto a computer monitor, which allowed for more accurate counting. Germination frequencies were obtained by taking the total number of pollen grains on the computer screen divided by the number of germinated pollen grains.

Pollen tube growth rate

The same plants were used from first study to determine pollen tube growth rates. Pollen was extracted daily at 9 am during anthesis. Pollen from each plant type (GFP-tagged pollen, 35s-GFP pollen, and wild type cv. 'Xanthi' pollen) was placed in Falcon plate wells and 100µl of BK media was added to three replicates per plant. Each replicate consisted of 5 wells each representing a specific hour (1-5 hrs). After one hour, 30ul of pollen/BK mixture was taken from the Falcon well and placed on a microscope slide. Pictures were taken with a digital camera (Olympus Q color 3) mounted on the microscope, after which, the pollen samples were discarded. At hour 2, 30µl of pollen/BK mixture was placed on a fresh microscope slide and photographed. This was repeated for a total of 5 hours. After each hour fresh pollen tubes were measured on the computer monitor with a ruler in cm and converted into mm by using a Hausser Scientific Brightline Hemacytometer with known grid sizes. The hemacytometer was placed on the microscope under 100X magnification where grid lines equal 0.05mm. Using a digital camera, the grid was projected onto the computer monitor and grid lines were measured with a ruler. The grid lines projected onto the computer monitor were equal to 1.5cm

therefore all pollen tube measurements were taken in cm from the computer monitor and converted into millimeters using this conversion. Pollen tube growth rate was calculated over a 5-hour period for the three tobacco types.

Results:

All pollen grains produced from the homozygous GFP-tagged pollen tobacco plants expressed GFP (Figure 4) as expected when viewed with an epifluorescent microscope under blue light. The GFP was also observed in the pollen tubes of GFP-tagged pollen GFP fluorescence was not observed in 35s-GFP or wild type tobacco pollen (Figure 5).

Average pollen tube germination frequencies were calculated using counts of the total number of pollen divided by the number of pollen grains that germinated after 3 hours (Table 1). GFP-tagged, 35s-GFP and wild type tobacco plants had an average pollen tube germination frequency of 52.7 ± 11.1, 57.1 ± 14.7, and 53.5 ± 11.2 respectively (Figure 6). An analysis of variance test was performed on average pollen tube germination for each tobacco type (GFP-tagged, 35s-GFP and wild type) to determine variation among type by day and by plant. Overall, there was no significant difference found among the average pollen tube germination frequencies of the 3 tobacco types ($P= 0.65$)

Pollen tube growth was observed over a 5-hour period (Figure 7). Measurements were taken per hour from a total of 6,242 grains of GFP-tagged pollen, 6,980 grains of 35s-GFP pollen, and 6,228 grains of wild type pollen (Table 2). Regression analyses were performed on the three tobacco types in order to calculate an average pollen tube

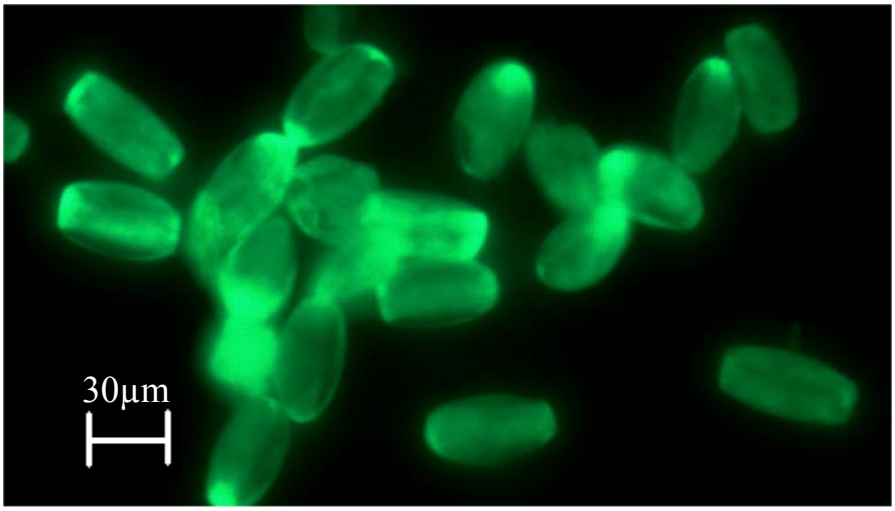

Figure 4. Dry tobacco pollen sample from homozygous GFP-tagged tobacco plants from the T_3 generation under 400x magnification. All pollen grains are expressing the GFP protein.

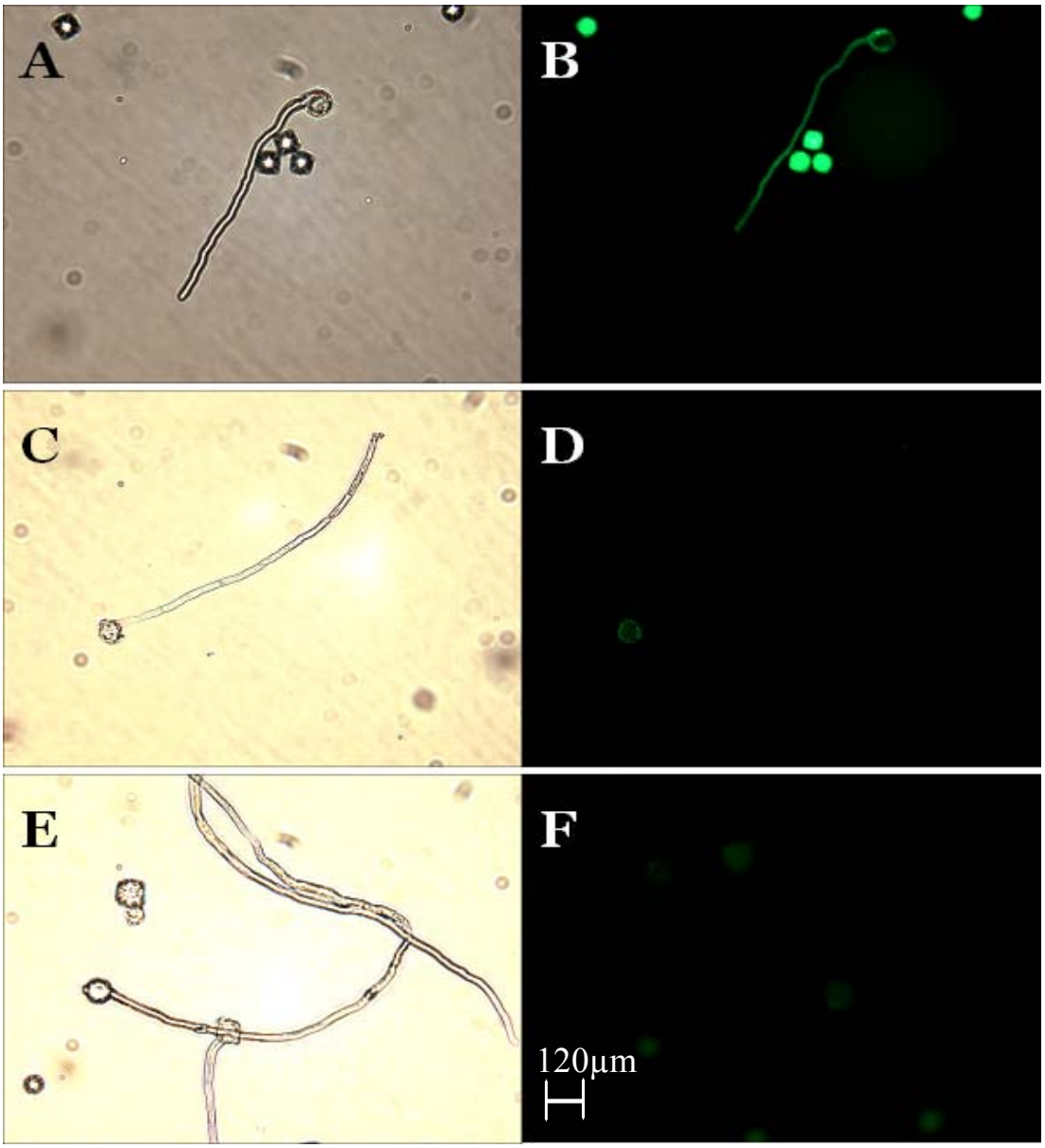

Figure 5. GFP is detectable in the pollen tubes. A and B show germinated pollen from GFP-tagged tobacco under white light (left) and blue light (right). C and D show germinated pollen from 35s-GFP tobacco under white light (left) and blue light (right) and E and F represent germinated pollen from wild type cv. 'Xanthi' plants under white light (left) and blue light (right). Pollen tubes were germinated in BK media and pictures were taken under 100X magnification with a 16ms exposure time under white light conditions (left), and 2.75s under blue light (right)

Table 1. Pollen grain germination frequency of three tobacco lines. The data were collected 3 hours post treatment with germination media, and represent five discrete samples from five different plants per line.

Type	Total number pollen grains	Number of pollen grains germinated
GFP-tagged	13,306	7,013
35s-GFP	13,807	8,080
Xanthi	13,678	7,606

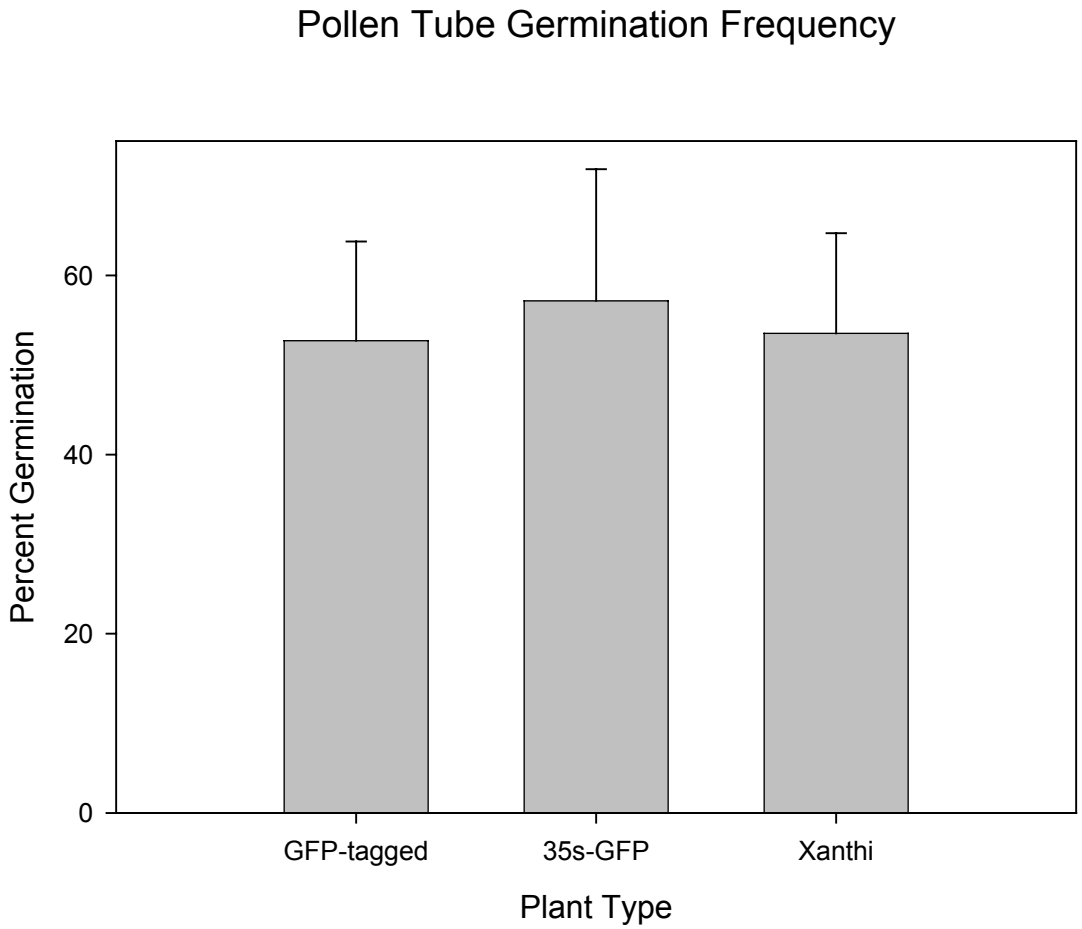

Figure 6. Average germination frequency (± SD) of pollen from 5 plants per type after 3 hours in BK pollen tube germination media. GFP-tagged pollen had a germination frequency of 52.7 ± 11.1, 35s-GFP had a frequency of 57.1 ± 14.7 and wild type tobacco had an average pollen tube germination frequency of 53.5 ± 11.2.

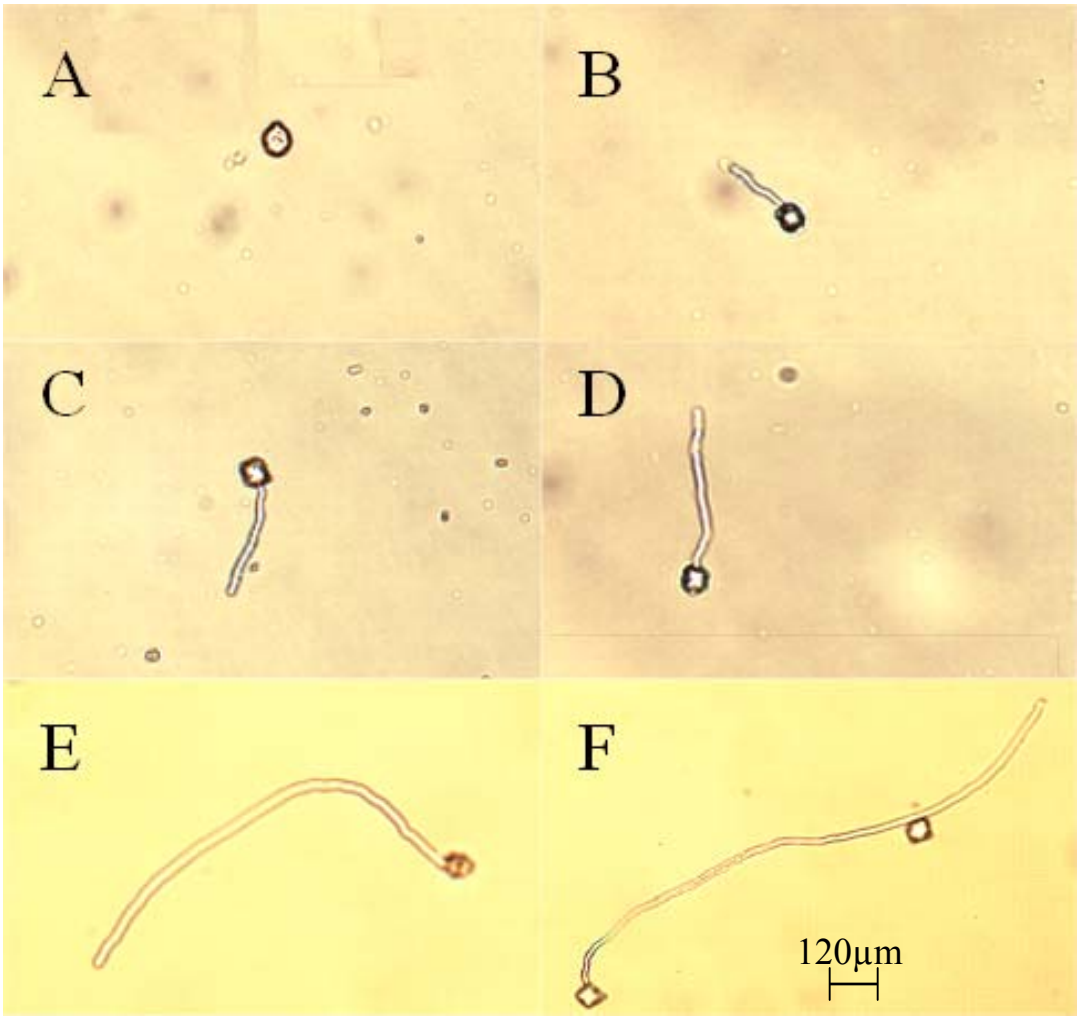

Figure 7. Tobacco pollen tube growth over a 5 hour time span. Photographs taken at 100X magnification with a 16ms exposure time under white light conditions. A) Tobacco pollen germination at addition of BK germination media. B) Tobacco pollen 1-hour post germination. C) 2 hour D) 3 hour E) 4 hour F) 5 hour.

Table 2. Total number of GFP-tagged, 35s-GFP and wild type 'Xanthi' tobacco pollen germinated. Pollen tube growth was measured per hour.

Type	Total number of pollen grains measured per hour				
	1h	2h	3h	4h	5h
GFP-tagged	1,500	1,500	1,243	962	1,037
35s-GFP	1,500	1,500	1,500	1,248	1,232
Xanthi	1,500	1,500	1,259	1,005	964

growth rate. Tobacco pollen from GFP-tagged pollen plants had an average tube length of 0.40mm ± .09 at 5 hours and an average growth rate of 0.09mm/hr (R^2=0.90). Pollen from 35s-GFP tobacco plants had an average pollen tube length of 0.41mm ± 0.09 at 5 hours and an average growth rate of 0.10mm/hr (R^2=0.98). Wild type tobacco pollen had an average tube length of 0.40mm ± 0.05 and an average pollen tube growth rate of 0.09mm/hr (R^2=0.95) (Figure 8).

Discussion:

The expression of green fluorescent protein under the control of the LAT59 pollen specific promoter in the pollen grains of *Nicotiana tabacum* cv 'Xanthi' does not pose a loss in pollen fitness according to the data reported in this study. Levels of GFP under the control of the LAT59 promoter are not toxic for the development or function of tobacco pollen. The data suggest that GFP can be used as a marker of gene expression in plant reproductive biology for studies addressing growth of pollen tubes through the style, fertilization events, and development of zygotic embryos. GFP can also be used as a marker in pollen. GFP pollen could be used to determine pollen movement under field conditions in order to assess possible gene flow risks of transgenic crops. In this experiment, green fluorescence was evenly distributed in the cytoplasm of all pollen grains from plants with the use of the variant *mgfp5-er*, which is targeted to the endoplasmic reticulum, under the control of the LAT59 pollen specific promoter in transformed homozygous tobacco plants. Fluorescence of GFP traveled out of the mature tube of GFP-tagged pollen. This was expected and is consistent with data indicating that

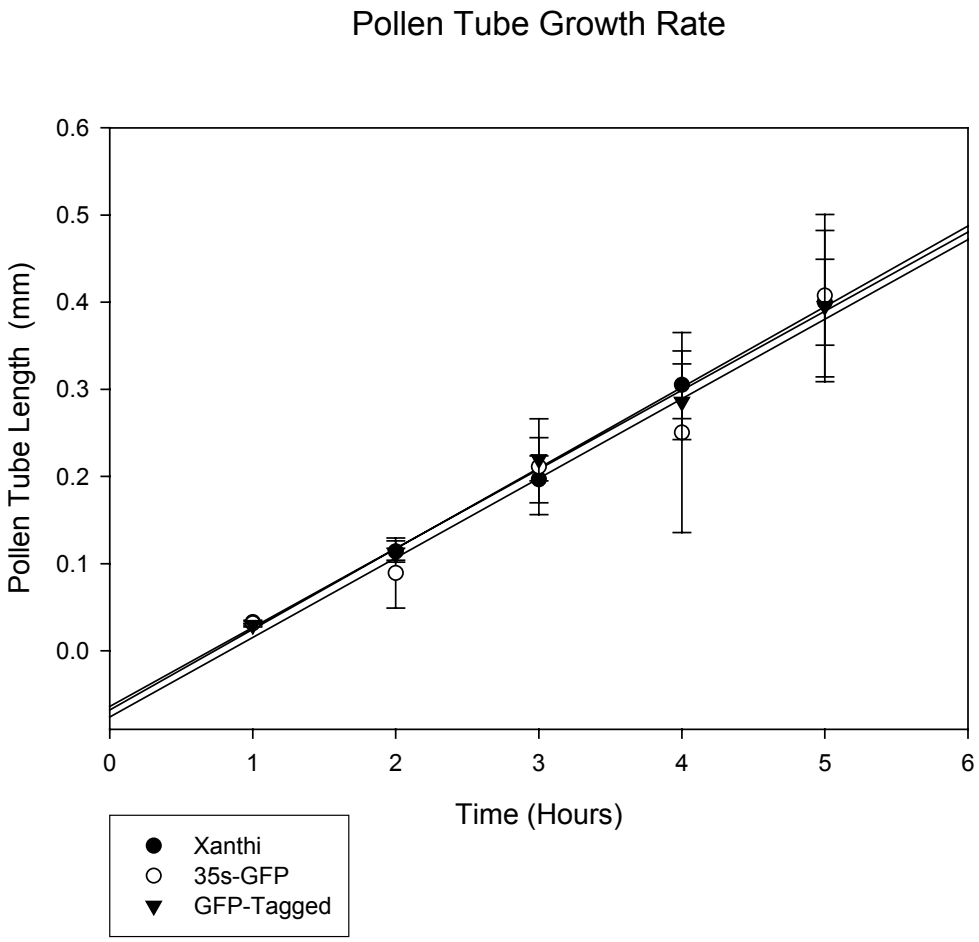

Figure 8. Regression of average pollen tube growth rates. Rates measured in mm after 5 hours in BK germination medium for GFP-tagged, 35s-GFP, and wild type cv. 'Xanthi' were 0.092mm/hr (R^2=0.90), 0.103mm/hr (R^2=0.98), and 0.094mm/hr (R^2=0.95). Sample was taken from 5 plants per type.

the LAT59 gene is expressed after microspore mitosis and reaches a maximum expression in mature microspores (Mascarenhas, 1990). The LAT59 gene also encodes a pectin-degrading enzyme (Wing *et al*., 1989) whose presence would be required for pollen tube synthesis and solubilization of cell wall material during the emergence and rapid growth of the pollen tube through the pistil.

High levels of GFP expression have been shown to have a detrimental effect on living cells (Liu *et al*., 1999) including pollen. Pollen showing very bright GFP fluorescence contained thick actin cables, showed slow cytoplasmic streaming, and tended to cease growth prematurely (Benedikt *et al*. 1998). On the contrary, Harper *et al*., (1999) showed that whole plant expression of GFP had no fitness costs to field grown tobacco plants. In this study, the presence of GFP in pollen did not impair reproductive function or cause any apparent toxic effects on pollen development, pollen germination, or tube growth. This is supported by previous work by Ottenschlager *et al*., (1999) who found no toxic effects with GFP expression driven by other pollen specific promoters in the pollen of several different plant species including tobacco. In this study average pollen tube germination frequencies of GFP-tagged pollen were not significantly different from that of pollen that was not fluorescent and there was little variation between plants or between the days from which pollen extraction took place. Pollen tube growth rates between the three types were statistically consistent and at 5 hours pollen tubes from each type had grown to approximately to the same lengths. GFP expression was not detected in 35s-GFP pollen as also observed in several previous studies that addressed gene expression in pollen cells under the control of the CaMV35s promoter (Twell *et al*., 1989; Stoger *et al*., 1992; Wilkinson *et al*., 1997; Mascarenhas and Hamilton, 1992).

The activity of transgene promoters in pollen could be important in agricultural applications of transgenic crops. These promoters could be used as a tool for the assessment of the implications that transgenic crops could impose on the environment. The LAT59 promoter isolates expression of GFP solely to pollen grains and could be used as a model system to track pollen movement in the field. In order to successfully implement GFP-tagged pollen as a model system it must act in the same way as the population that you intend to study. To answer this question, two parameters must be addressed. Determination of the viability of transgenic pollen is important since pollen flow is the means for which out-crossing events can occur under field conditions. Secondly, the determination of any fitness enhancements or costs associated with transgene expression in pollen that could cause it to out compete or be out competed by wild type pollen is also important. GFP has no effect on tobacco pollen according to this study, and can be easily distinguished from wild type pollen. This makes GFP-tagged pollen a good candidate for use in a model system to determine out-crossing distances by tracking pollen flow within a field, or to determine any effects that the presence of a non-endogenous protein in pollen could have on pollinating insects.

Chapter Four

The Use of Green Fluorescent Protein for Detection of Gene Flow and Pollen Distribution

Abstract:

The incorporation of transgenic crops into agricultural systems has increased the potential of pollen-mediated transgene escape into the natural environment. Potential consequences of gene flow warrant the need for a system to detect gene flow and pollen movement. Transgenic tobacco has been developed for use as a potential marker for gene flow. I have shown the use of whole plant fluorescence to determine out-crossing events and have developed a model system using GFP-tagged pollen to characterize the spatial distribution patterns of transgenic plant pollen in the environment.

Gene flow and pollen movement were quantified under field levels. The field design consisted of a center plot split into 4 quadrants (2 plots of GFP-tagged pollen plants and 2 plots containing plants expressing GFP constitutively). Wild type receptor plants and pollen traps were placed in a spatial grid around the center plot. Progeny from wild type plants were screened with a hand held ultraviolet light for detection of constitutive GFP expression. Pollen traps were collected and screened for presence of GFP-tagged pollen using fluorescent microscopy. Progeny from recipient plants outside of the donor plot were screened for the GFP phenotype and percent out-crossing of GFP tobacco was 0.009% in 2001 and 0.07% in 2002. Progeny from donor plants within the center plot were screened for the GFP phenotype in 2002 and average out-crossing of GFP tobacco

was 0.7%. Out-crossing frequencies among quadrants were not significantly different (T test, $P > 0.05$).Presence of tobacco pollen was undetectable on pollen traps from the field experiments. While pollen flow from tobacco was undetectable, out-crossing was occurring. Since tobacco is predominately self-fertilizing, a more ecologically important crop such as canola might be a more appropriate model. GFP-tagged pollen has the potential for risk assessment research and might be an enabling tool for in the monitoring of transgenic crop pollen flow in agronomic systems.

Introduction:

Over the past decade the use of molecular techniques in plant breeding has lead to the widespread use of transgenic crops in our agricultural system. These technological advances present new opportunities for developing plants that are resistant to pests and disease, better able to withstand stressful environments, and have the capacity to produce better quality food products. As with most significant advances in biological science, concerns are raised about the potential consequences of these developments to the environment. One of the principal concerns of genetically modified crops is the likelihood and possible consequence of the introduced transgenes being transferred by cross-pollination to wild populations of plants growing in close proximity. Interspeific hybridization between crop species and their wild relatives is a valid concern considering that 12 out of the 13 most important crops grown worldwide are sympatrically located to crossable relatives somewhere in their cultivation area (Ellstrand *et al*.,1999). Transgenic crops growing in close proximity to wild relatives could create conditions for unwanted hybridization. This is the case, among others, of maize (Doebley, 1990), canola (Klinger

et al., 1992), sunflower (Whitton *et al*., 1997) and sugar beet (Bartsch and Pohl-Orf, 1996).

In order for pollen mediated gene flow to occur among plant populations, dispersal of pollen to a different population must occur with successful fertilization of an ovule. Previous attempts to measure gene flow have evolved around the analyses of genetic markers (Slatkin, 1985) that uses population genetic structure gathered from isozyme surveys which can be fit to data models of population differentiation (Slatkin and Barton, 1989). This system has limitations because it provides no absolute measure of gene flow, and is unable to distinguish between gene flow via pollen from that via seed. Other methods have revealed gene flow by paternity analysis using microsatellite markers (Dow and Ashley, 1998) however this method requires the use of expensive molecular techniques to analyze seeds for the presence of microsatellite loci. More recently, visual markers such as GFP have been proposed for use, using whole plant expression to monitor gene flow under agricultural conditions (Stewart, 1996; Leffel *et al*., 1997; Harper *et al*., 1999). This method has been used successfully to assess out-crossing events in canola (*Brassica napus*) under field conditions (Halfhill *et al*., 2003).

A complete description of gene flow in plants must include an assessment of the relative importance of pollen as the agent of gene flow. Currently, there are few systems for the direct monitoring of pollen movement. Other research approaches have concentrated exclusively on gene flow (Ellstrand, 1992) by calculating migration rates as the proportion of foreign pollen among successful pollen using paternity exclusion analysis (Ellstrand *et al*., 1989; Friedman and Adams, 1985; Adams and Birkes, 1990).

Due to the complexity of these methods there is a need for an efficient monitoring system to detect transgenic pollen flow so that potential risks can be quantified and evaluated.

A more simple method could be GFP-tagged pollen to monitor pollen movement under field conditions directly. GFP expression in plant pollen will not only enable the tracking of pollen movement, but also can be used to differentiate between pollen from individual plants of the same species. GFP-tagged pollen could also be used to assess pollination mechanisms, and spatial patterns of pollen distribution.

This study had two goals. The first goal was to determine pollen-mediated gene flow of transgenic tobacco to non-transgenic tobacco at various distances from a source population using whole plant expression of GFP. Gene flow was determined by screening progeny from wild type recipient plants in order to evaluate the adequacy of current isolation distances for the prevention of out-crossing. Secondly, I describe proof-of-concept experiments in *N. tabacum* (tobacco) using GFP-tagged pollen (Hudson *et al*., 2001) showing the potential of a system to detect pollen movement under field conditions. This system would allow the direct quantification of pollen flow directly from a group of individuals in the field and to determine distance and directional patterns of pollen dispersal within a tobacco population.

Materials and Methods:

Plant material

The field design of this experiment incorporated two types of transgenic tobacco. Tobacco plants expressing GFP through out the entire plant (WPGFP) contained the *mgfp5-er* transgene, driven by the cauliflower mosaic virus 35S (CaMV35S) constitutive

promoter. WPGFP tobacco plants were used to measure gene flow in the field. Homozygous WPGFP seeds were germinated on MS medium containing kanamycin 200mg/L as a selection agent. After germination, plantlets were transferred to soil and the phenotype was confirmed by GFP visualization with a hand-held, long wave UV light (UVP model B-100AP 100 W: 365nm). Plantlets were placed in the greenhouse until transferred to the field sites.

GFP pollen specific tobacco plants (PGFP) expressed the *mgfp5-er* transgene, driven by the LAT59 pollen specific promoter. PGFP tobacco plants expressed the GFP protein exclusively within pollen grains and were used to measure pollen movement in the field. Homozygous PGFP seeds (T_2) were germinated on MS media with kanamycin 200mg/L. After germination, plantlets were placed in soil and grown in greenhouse conditions until planted at the field sites.

Field design

Field experiments were conducted at the Upper Piedmont Research station in Reidsville, NC in the summer of 2001 and the Tennessee Small Grains Unit of the Knoxville Experimental Station in Knoxville, TN, USA in the summer of 2002. The experimental field design was based on Saeglitz *et al*., (2000). The field design consisted of a central donor plot split into four quadrants. Two quadrants contained WPGFP tobacco plants. The remaining two quadrants of the center donor plot contained PGFP tobacco plants. Each of the 4 quadrants contained 6 rows with 8 tobacco plants per row giving a total of 192 transgenic tobacco plants located within the center donor plot. Two wild type recipient tobacco plants cv 'Xanthi' were placed in a spatial grid around the center donor plot in 8 directions at distances of 10 to 100m from

the center plot (Figure 9) A pollen trap was placed in 8 different directions (N, S, E, W, NW, SW, NE, SE) at distances of 5, 10, 15, 20 and 25m meters from the center donor plot as well as inside each quadrant within the center plot to measure wind dispersed pollen flow.

Gene flow data collection

North Carolina 2001

Seed capsules were harvested from the receptor plants, which surround the donor plot at various distances and directions. Seeds were germinated under greenhouse conditions from each receptor plant. Gene flow was quantified under field conditions by progeny analysis for the GFP phenotype with a hand held UV light (Figure 10). Plants expressing GFP were confirmed with fluorescent spectrophotometry (Leffel *et al*., 1997; Harper *et al*., 1999; Niwa *et al.,* 1999; Halfhill *et al*., 2001) using a FluoroMax-2 (Jobin Yvon & Glen Spectra, Edison, NJ) with Datamax spectroscopy software (Galactic Industries, Salem, NH, USA). Leaf samples were excited with FluoroMax-2 at 385nm and emission spectra were recorded from 420 to 560 nm and intensity was measured at 508nm (green light) in counts per second (cps)(Millwood *et al*., 2003). Out-crossing frequencies were calculated from the summed progeny at each coordinate and represent the average out-crossing frequency per plant.

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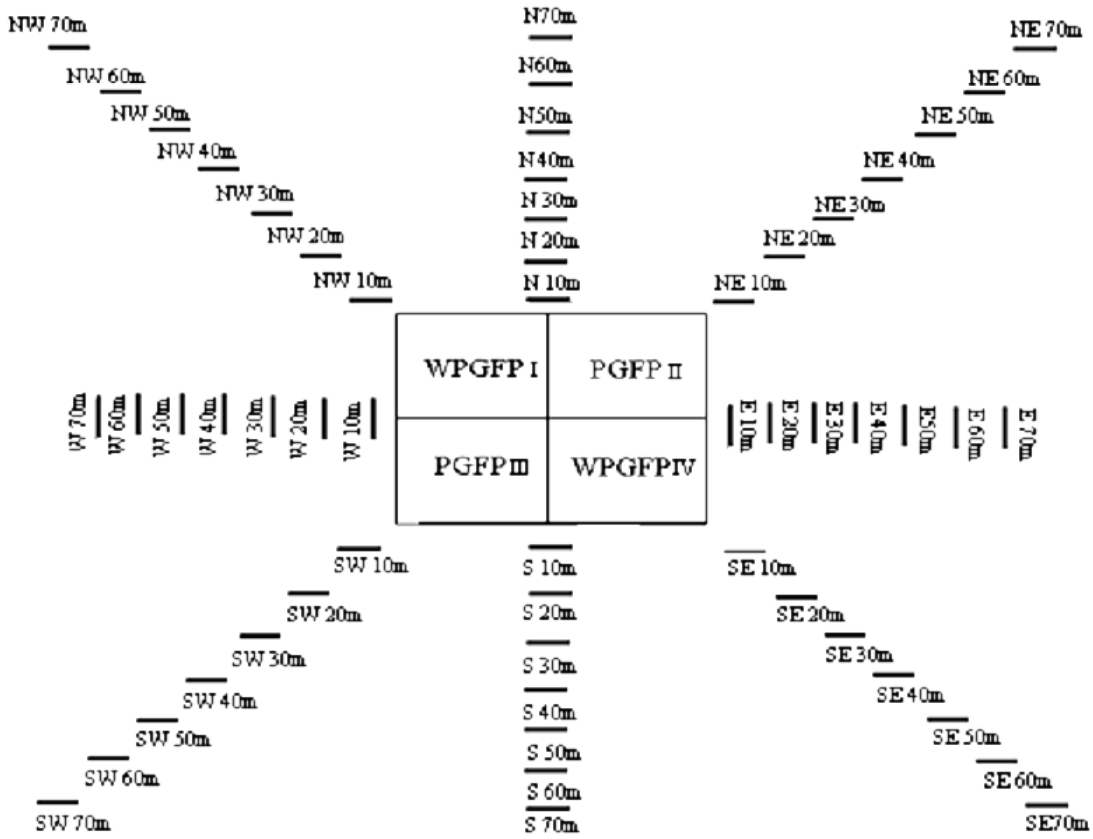

Figure 9. The experimental field design. The design consists of a central plot split into 4 quadrants. Two quadrants contain GFP pollen specific plants (PGFP), and the remaining two contain whole plant-fluorescent GFP plants (WPGFP). Two wild type tobacco plants cv 'Xanthi', along with a pollen trap, were placed in 8 directions of the compass (N, NW, NE, S, SW, SE, E, and W) at distances of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 meters from the center plot. Not to scale.

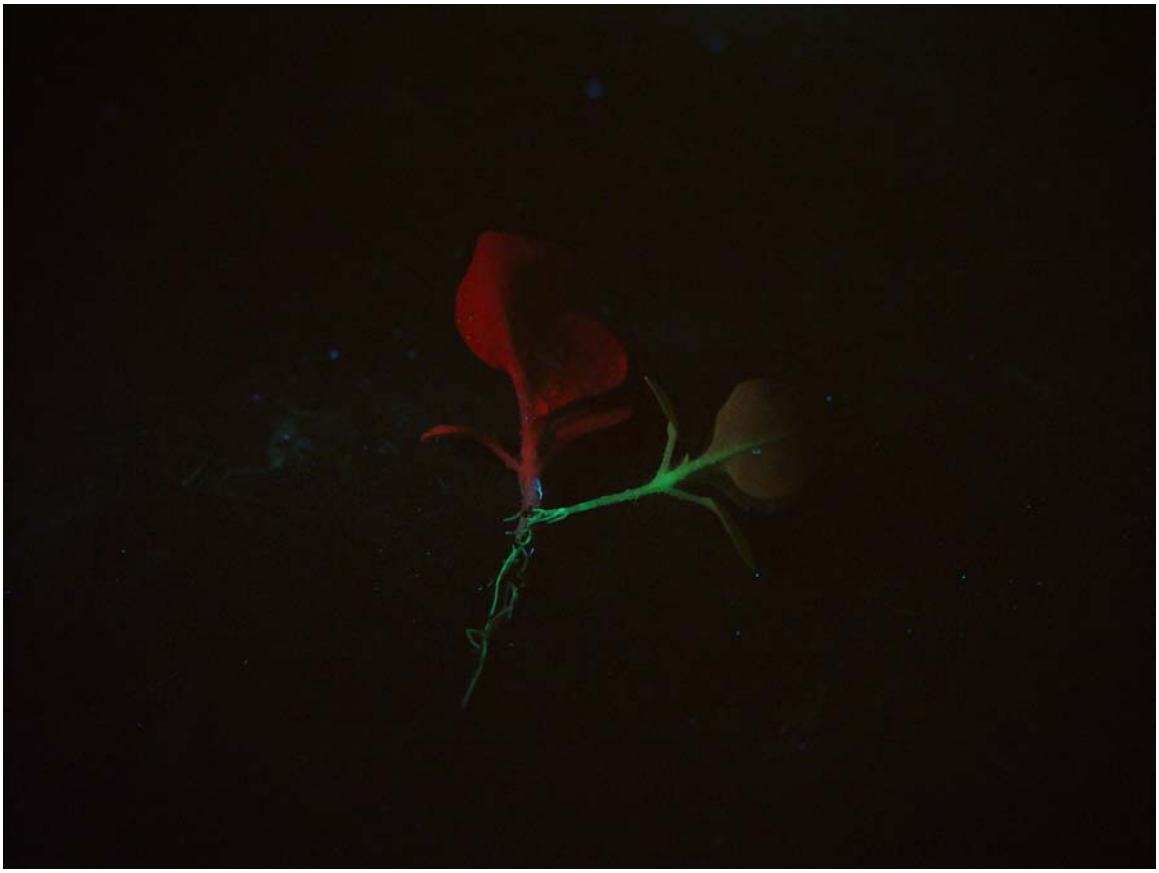

Figure 10. GFP phenotype compared to wild type. Constitutive expression of the GFP phenotype (right) in tobacco seedlings shown under a hand held, long wave UV light when compared to wild type cv. 'Xanthi'(right).

Tennessee 2002

Seed capsules were harvested from the receptor plants as mentioned previously. Seeds from these plants were germinated in a dark incubator at 27°C on filter paper soaked in a calcium chloride solution (0.2g/L). After 3 weeks gene flow was quantified by progeny analysis of seedlings from recipient plants and plants expressing GFP in the pollen (PGFP) within the donor plot. Seedlings were screened for the GFP phenotype with a hand held UV light. Out-crossing frequencies were calculated from the summed progeny at each coordinate and represent the average out-crossing frequency per plant.

Pollen flow detection

Along with gene flow, wind dispersed pollen flow was measured in the two field experiments. Pollen flow was measured with pollen traps in order to sample pollen distribution at specified distances. Replicate pollen traps were constructed from double-sided sticky adhesive tape on glass microscope slides. Slides were covered with petroleum jelly and attached to vertical wooden stakes with collection heights of 50cm and 100cm from the soil surface. Pollen dispersion was measured from the onset of anthesis to 24, 48, and 72 hour periods after pollen shed from the donor tobacco population within the center plot. Presence of GFP-tagged pollen was assessed by fluorescent microscopy using an epifluorescent microscope with blue light at 100x magnification without staining.

Results:

Out-crossing frequencies in *Nicotiana tobacum* under field conditions

Over the two-year field trials out-crossing occured at long distances, but at low frequencies within the fields of tobacco. In North Carolina 2001, progeny analysis found a single out-crossing event occurring at 3 different coordinates located 70m north, 20m south and 20m west of the center donor plot. A total of 37,840 seedlings were screened for the GFP phenotype yielding a total out-crossing frequency of 0.0079% (Table 3) for that year. Progeny positive for the GFP phenotype located 70m north, 20m south and 20m west were confirmed using fluorescence spectrophotometry analysis at 508nm emission peaks of 5.1cps, 3.9cps, and 4.6cps respectively and a wild type emission of 2.6cps (all units in 10^5 counts per second) (Figure 11).

In Tennessee 2002, a single out-crossing event was detected from a recipient plant located at coordinate 10m north of the center donor plot. A total of 2,778 seedlings were screened for the GFP phenotype with a total out-crossing frequency of 0.07% (Table 4) occurring from recipient plants out side of the donor plot.

Out-crossing occurred at a slightly higher rate within the center donor plot than at surrounding coordinates. Within quadrant PGFPII, in the center donor plot, seeds were collected from 12 plants. From these seeds 31 plants were found to be expressing GFP out of a total of 2,696 seedlings screened from that quadrant. The average out-crossing frequency for quadrant PGFPII was 0.97 ± 1.27. Seeds from 16 plants were collected from quadrant PGFPIII from which 2,966 seedlings were screened. The GFP phenotype was found in 11 of these progeny. The average rate of out-crossing for quadrant PGFPIII was found to be 0.32 ± 0.73 (Table 5).

Table 3. Average out-crossing of GFP tobacco in Reidsville, North Carolina, USA, 2001. Progeny from recipient plants outside of the donor plot were screened for the GFP phenotype. Plantlets from each coordinate were summed and out-crossing frequencies represent the average per plant at each coordinate.

Coordinate Location	Seedlings Screened	Number Transgenic	Out-Crossing Frequency
N 70m	346	1	0.14 ± 0.20
S 20m	1,027	1	0.10 ± 0.14
W 20m	754	1	0.14± 0.19
All Other Coordinates	35,713	0	0
Totals	37,840	3	0.0079%

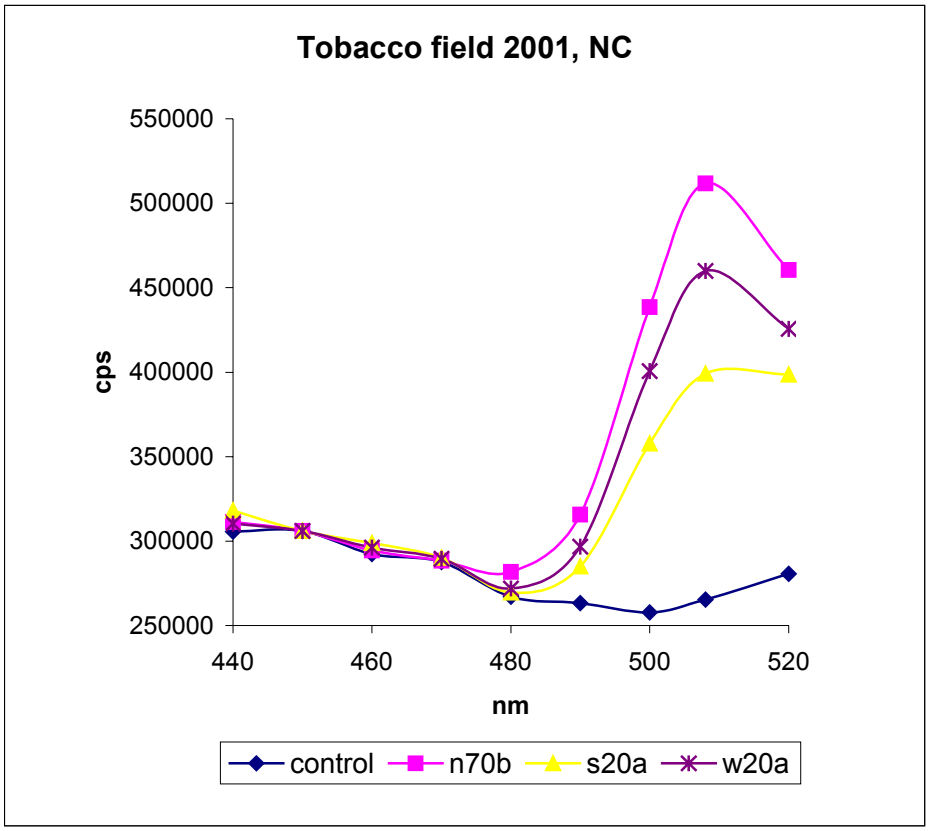

Figure 11. Spectrophotometry analysis**.** Leaf samples from putative out-crossing events from coordinates North 70m, South 20m, and West 20m from the field site in Reidsville, NC were excited with a FluoroMax-2 with Datamax spectroscopy software at 385nm and emission spectra were recorded from 420 to 560 nm and intensity was measured at 508nm (green light) in counts per second (cps)

Table 4. Average out-crossing of GFP tobacco in Knoxville, TN, USA, 2002. Progeny outside the donor plot were screened for the GFP phenotype. Plantlets from each coordinate were summed and out-crossing frequencies represent the average per plant at each coordinate.

Coordinate Location	Seedlings Screened	Number Transgenic	Out-Crossing Frequency
SW 10m	148	1	0.34 ± 0.48
E 10m	40	1	1.25 ± 1.77
All Other Coordinates	2,590	0	0
Totals	2,778	2	0.0719%

Table 5. Average out-crossing within the center plot of GFP tobacco in Knoxville, TN, USA in 2002. Progeny within the donor plot were screened for the GFP phenotype. Seedlings screened and number transgenic represent the sum of all plants within each quadrant and out-crossing frequency represents the average per plant within each quadrant. Out-crossing frequencies among quadrants PGFPII and PGFPIII were not significantly different (T test, P= 0.13)

Coordinate Location	Number of Plants	Seedlings Screened	Number Transgenic	Out-Crossing Frequency
PGFPII	12	2,696	31	0.97 ± 1.27
PGFPIII	16	2,966	11	0.32 ± 0.73
Totals	28	5,662	42	0.742%

Pollen flow detection under field conditions

Wind dispersed pollen flow was measured using pollen traps in order to sample pollen distribution at specified distances. Eighty-eight pollen traps were collected each year in North Carolina and in Tennessee beginning at the onset of anthesis to 24, 48, and 72 hour period after pollen shed from the tobacco population within the center plot. The presence of tobacco pollen, transgenic or wild type was undetectable on pollen traps from field experiments performed over the two-year trial.

In the North Carolina field trial, bees were captured at approximately 20m southwest, 10m north, 60m west and within the PGFPIII plot and screened for the presence of GFP pollen. The presence of GFP pollen was undetectable using an epifluorescent microscope with blue light on the insects captured under field conditions.

Discussion:

Literature on the out-crossing of tobacco is not extensive because it is considered to be highly autogamous. However, a range of out-crossing rates has been detected under field conditions. Out-crossing frequencies of tobacco have been found to vary between variety, season, and location and range from 0.2 to 18% (Hays *et al*., 1927; Krishnamurty, 1958; Litton and Stokes,1964; McMurtrey *et. al*., 1959). In this study out-crossing occurred under field conditions in tobacco over a two-year field trial. Gene flow occurred at low frequencies at five coordinates. This is consistent with other predominately self-pollinating species such as soybean (*Glycine max* L.), which has out-crossing rates of less than 1% (Garber and Odland, 1926; Chiang and Kiang, 1987). Four out of the five hybridization events found over the two-year field study occurred within

30 meters from the center donor plot of transgenic plants. This is consistent with other research that has shown out-crossing distances in tobacco to occur at 21m (Paul *et al*., 1991) This low hybridization frequency found in the recipient tobacco population was in contrast to higher out-crossing frequencies found to occur within the center donor population itself as seen in other pollen movement models (Faegri and van der Pijil, 1979; Whitehead, 1983; and Luna *et al*., 2001). A single out-crossing event was found at a distance of 70m from the center plot. This can be considered a long distance for a species that is characteristically self-fertilizing when compared to known out-crossing species with a wide range of hybridization distances varying from barley at 100m (Ritala *et al*., 2002), wild radish at 650m (Ellstrand *et al*., 1985), and canola at 550m (Hall *et al*., 2000). Other research groups have found out-crossing in tobacco populations to occur at long distances ranging from 82 to 727m (McMurtrey *et al*., 1959). The maximum distance of out-crossing from this study was within the range of 375m, which is considered adequate for the production of certified seeds. Based on the data collected from these field trials no evidence of a directional pattern of pollen movement could be determined.

Pollen expressing the GFP protein could be used to detect pollen movement via pollinators in the field. It is difficult to deduce the direction and distance of gene flow by pollen as assisted by bees. As with genetic markers used to detect pollen flow by pollinators using paternity testing of seed embryos (Jackson, 1996) or through isozyme markers (Jackson and Clark, 1991) visual detection of GFP pollen on pollinators proved to be a difficult task. It is possible to visualize GFP pollen on the body of a pollinating insect (Hudson *et al*., 2001), however under field condition, that becomes problematic.

Pollinators, such as bees, are known to travel 2 to 3 miles from their hives in order to forage nectar and pollen from flowering plants (Jackson, 1996). Bees captured at the North Carolina field site were carrying large pollen loads from plants of a variety of species making it difficult to visualize tobacco pollen specifically within this pollen load using microscopy. To add to the difficulty, these loads of pollen are compacted together by the insect to form a pellet for easy transport. Pollen has a slight autofluorescence when exposed to blue light under the microscope. A large pollen load emits a combined autofluorescence making it problematic to visualize GFP-tagged pollen on an insect using this method. Pollen flow from tobacco was undetectable when monitoring for the presence of GFP-tagged pollen on pollen traps. However, out-crossing was occurring, confirming pollen movement within the field site. Pollination can be studied by following the physical movement of pollen using traps (Greenwood, 1986; Caron and Leblanc, 1992) however, it is possible that pollen traps used for this experiment were not ideal and could be modified for future studies of this application of GFP-tagged pollen. Tobacco is not an ideal model for development of a system to detect pollen movement by GFP fluorescence.

The GFP-tagged pollen system might be more useful for monitoring pollen flow in an out-crossing species such as canola. Canola is a better candidate considering that it is a known out-crosser and potential pollen movement has been documented more than 500m from the pollen donor source (Hall *et al*., 2000). This is greater than the 100m isolation distance currently regulated for seed growers. These large distances may be problematic since transgenes can flow to weedy relative populations (Timmons *et al*., 1996; Mikkelsen *et al*, 1996). Canola has a high percentage rate of self-fertilization however it

is both insect and wind pollinated. Canola pollen can become air born allowing out-crossing to occur at greater distances than in tobacco. Efforts are currently under way to transform canola cv. 'Westar' to be used as a candidate for the GFP-tagged monitoring system in the field. GFP-tagged pollen has the potential for use in risk assessment research and might be an enabling tool in the commercial monitoring of transgenic crop pollen flow in the future.

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Vita

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